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Monoclonal Immunoglobulin

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Additional information is available at the end of the chapter

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1. Introduction

Secretion of monoclonal immunoglobulins (M-Ig) may be associated with several malignant conditions, also called M-protein, paraprotein, or M-component they are produced by an abnormally expanded single ("mono-") clone of plasma cells in an amount that can be detected in serum, urine, or rarely in other body fluids [1]. The M-Ig can be an intact immunoglobulin (Ig) (containing both heavy and light chains), or light chains in the absence of heavy chain (encountered in light chain myeloma, light chain deposition disease, AL amyloidosis), or rarely heavy chains in the absence of light chains only (heavy chain disease).

All intact Igs have the same structure, made up of mirror imaged identical light and heavy chains. There are five classes of heavy chain, γ , α , μ , δ and ϵ with two classes of light chain κ and λ . Igs are secreted by terminally differentiated B-lymphocytes and their normal function is to act as antibodies recognizing a specific antigen.

During B-cell maturation, the rearrangement of Ig heavy and light chain genes takes place early in pre-B-cell development and ends in memory B-cells or Ig producing plasma cells that have a unique heavy and light chain gene rearrangement, thus being selected to recognize a given antigen. During, oncogenic events which occur randomly during this process, the B cell may acquire a survival advantage, and proliferate into identical (clonal) daughter B-cells able to differentiate into Ig producing cells secreting a monoclonal component. With additional oncogenic events a mature B-cell neoplasm may develop, carrying the inherent ability to produce a monoclonal Ig. Multiple myeloma and Waldenstrom's macroglobulinaemia are archetypical of Ig-secreting B-cell disorders.

The purpose of this present chapter is to describe the properties of M-Igs and discuss the biologic, clinical and other implications of their presence in the course of B-cell disease entities.

2. Ontogeny of normal and monoclonal Ig-producing B-cells

2.1. B-cell development

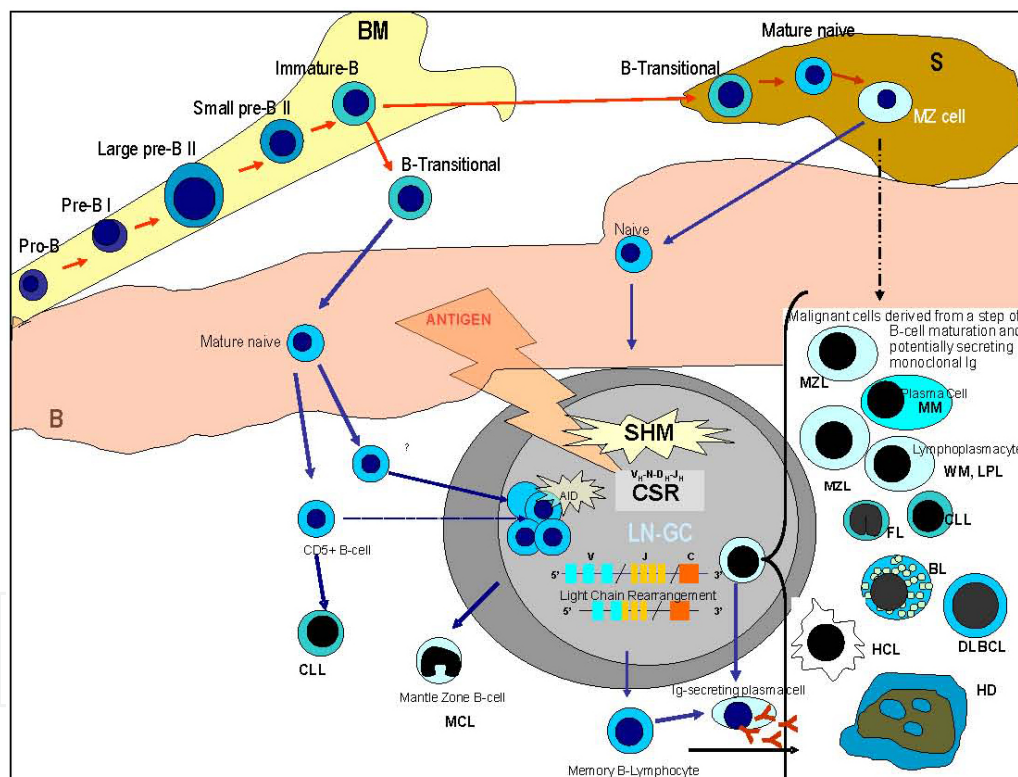
B-cell maturation is a complex process that comprises both cell differentiation into Ig secreting plasma cells and, in parallel, the rearrangement of the genes responsible for Ig synthesis. Furthermore it includes inherent risks of genetic derailment because it is associated with DNA remodelling with intrinsic instability, thus presenting the possibility of malignant development.

B cell development begins in the bone marrow (BM) from gestation week 18 and throughout life. The generation of pro-B cells from a common lymphoid progenitor cell depends on two main transcription factors, E12 and E47 and on the contribution of the transcriptional regulators EBF and Pax-5 [5]. During B-cell evolution the rearrangement of Ig heavy and light chain genes takes place [2]. The Ig heavy gene (IgH) is located on chromosome 14 while Ig light chain (IgL) genes are on chromosomes 2 and 22 for κ (1-40 $\nu\kappa$, 1-5 $j\kappa$ and 1 $\kappa\kappa$) and λ (1-30 $\nu\lambda$, 1-4 $j\lambda$ and 1-4 $c\lambda$) light chain respectively. Rearrangement of IgH and IgL genes allows variable (V), diversity (D) and joining (J) gene segments rearrangement. V(D)J recombination starts in precursor B cells (pre B-I); recombinase activating genes 1 and 2 (RAG-1 and RAG-2), are essential for this step. The resulting IgVH is frequently not functional therefore the pre-B cell initiates V(D)J recombination at the other allele. If this is successful, the complete IgVH will be expressed as an Ig μ H chain in the cytoplasm (Cy-Ig μ) and on the membrane, together with a surrogate light chain, the pre B cell receptor complex (pre-BCR). Accordingly the pre-B-II cell proliferates, then loses its pre-BCR and re-express RAG proteins [7]. At that point, the B-cell is transformed into a small pre B-II cell that will subsequently rearrange the IgL variable gene segments and expresses a mature membrane BCR. If the BCR is not strongly self-reactive, the immature B cell leaves the BM as transitional B cell that evolves into naive B cell in the spleen; alternatively, it may mature in the periphery. However, if the immature B cell is still self-reactive, it will remain in the BM for additional IgVL recombination, replacing the self-reactive IgVL by another IgVL and so on. B cells producing self-reactive BCRs are removed from the repertoire during maturation by BM silencing mechanisms [3;4]. Splenic transitional B cells (CD27⁻ CD5⁺ CD10⁺ CD24^{hi} CD38^{hi} and L-selectin^{lo}) undergo differentiation into mature naive B2, also called follicular (FO) B cells, or marginal zone (MZ) B cells [5]. The aforementioned B-cell population is characterized by limited proliferative capacity and survival upon BCR stimulation; it comprises less than 2% of the peripheral B cells [6]. While maturing in the spleen, transitional B cells lose CD10 and CD5 and start expressing higher levels of L selectin and CD44. Following which the B cell transforms into conventional naive B2 cells that recirculate via the blood to the secondary lymphoid tissues or organs [7]. MZ cells could represent the normal counterpart of marginal zone lymphoma cells and CD5⁺ B-cells the one of mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL). Blood also contains a small normal population of naive CD5⁺ CD27⁻ cells that frequently produce poly-/self-

reactive antibodies (Abs) [8]. The CD5 molecule negatively regulates BCR signals [9] and CD5 B cells represent 50% of poly-/self-reactive cells [10].

Lymph node (LN) colonization depends on the expression of L-selectin and integrin $\alpha\text{L}\beta 2$ (LFA-1), while recruitment to mucosa-associated lymphoid tissues (MALT) depends on expression of L-selectin and integrin $\alpha 4\beta 7$. Without antigenic stimulation, the naive B cells recirculate again.

Activation of mature naive B cells into Ig secreting plasma cells can be T-helper independent (TI) and antigen free, via invariant receptors (TI-1), or derives from crosslinking of the BCR by polyvalent Ags (TI-2). More frequently, it is performed in close collaboration with CD4-expressing T cells (T-helper dependant: TD), and results from a monovalent Ag aggression. MZ B cells of the spleen and other mucosal sites, mostly respond to TI-2 Ags, such as polysaccharides of bacterial cell walls and other bacterial components, able to crosslink BCRs [11]. IgM+ MZ B cells that are CD27+ are memory cells while CD27- are naive; their BCRs display poly- and self- reactivity.



BM: Bone Marrow, S: Spleen, B: Blood, LN-GC: Lymph Node-Germinal Center, MZL: Mantle Cell Lymphoma, MM: Multiple Myeloma, LPL: Lymphoplasmacytic Lymphoma, WM: Waldenstroms Macroglobulinaemia, FL: Follicular Lymphoma, CLL: Chronic Lymphocytic Leukemia, BL: Burkitt Lymphoma, HCL: Hairy Cell Leukemia, DLBCL: Diffuse Large B Cell Lymphoma, HD: Hodgkin Lymphoma, SHM: Somatic Hypermutations, CSR: Class Switch Recombination.

Figure 1. Schematic of B-cell Maturation and B-Lymphoproliferative Disorders Origin

T-helper-cell dependent (TD) B-cell activation takes place in germinal centers (GC) in response to the presence of free Ags, as part of immune complexes or at the surface of Ag presenting

cells (APC). B-cells then differentiate into short-lived, Ab-forming plasma cells or proliferate as centroblasts expressing CD10+, CD38+ and BCL-6. These centroblasts express low amounts of the BCR at their surface and undergo somatic hypermutations (SHM), by accumulating nucleotide substitutions in their Ig variable (*IgV*) genes [12;13]. GC activated B-cells are meant to be short-lived, except for the few with a high affinity *IgV* region (BCR) for the Ag. These high-affinity B cells are selected in the GC light zone, and may undergo class switch recombination (CSR), switching the *IgM/IgD* sequence with any of the other downstream region sequences [14]. Igs formed early in the context of normal response to an Ag aggression are of *IgM* and *IgD* isotypes; these are located on the B-cell surface as recognition receptors. Then activated B cells divide, and class switching from the *IgD* and *IgM* heavy chains to *IgG*, *IgE* or *IgA* classes takes place [15;16]. The process is regulated by various cytokines [16] while both SHM and CSR depend on the B-cell-specific enzyme activation-induced cytidine deaminase (AID) which is highly expressed by GC B cells [17]. Cytokines and costimulatory soluble factors stimulate the transcriptional activation of individual I promoters and determine the S region and Ig isotype involved in the CSR event. SHM depends on transcription of the variable (*IgVH* and *IgVL*) regions and leads to point mutations and, to a lower extent, insertions and deletions. The rate of SHM is about 1 mutation on 1000 nucleotides per cell division. CSR consists on transcription of the S regions that started upstream of an I exon that is located 5' of each S region, giving rise to non-coding germline transcripts that span the I exon, the S region and downstream CH exons [7].

Terminally differentiated B cells become either Ab-producing mature plasma cells that home to the bone marrow or memory cells [18]. Memory B cells (CD27+) are Ag-selected B cells, derived from TD GC responses and usually express either *IgM*- *IgD*- or *IgM*+ *IgD*+, comprising about 20% of all peripheral B cells. A small percentage of *IgM* only (*IgM*+ *IgD*-) and *IgD* only (*IgM*- *IgD*+) also exists. *IgD*-only B cells have undergone a $C\mu$ deletion due to a non-canonical CSR event, express *Igλ*, contain extremely high levels of somatic *IgV* mutations [19] and show a strongly biased V3-30 *IgVH* gene usage [20], that can be seen in some malignant B-cell disorders [2]. Memory B-cells are long-lived, prone to Ig class switch (to *IgG*, *IgA* or *IgE*) and contain hypermutated *IgV* genes. Following stimulation, they present a competitive advantage over naive B cells in rapidly transforming themselves into plasma cells producing high affinity, class switched, *IgG/IgA* Abs [21]. They may hide in BM niches and recirculate numerous times. It is believed that in most indolent B-cell lymphoproliferative disorders, a proneoplastic condition precedes where the precursor neoplastic B-cell circulates and recirculates as a memory cell.

2.2. Malignant transformation

Where one or more oncogenic events occur during B-cell maturation, the resulting daughter cell will be identical and, if it has the ability to differentiate into an Ig producing cell, it will secrete a monoclonal component. Consequently, all B-cell mature neoplasms [22] have a common origin as well as the inherent ability to produce a monoclonal Ig.

Malignant B-cell Non-Hodgkin's lymphoma (NHL) possibly develops because risks for genetic derailment are increased during SHM and CSR that are associated with DNA remod-

elling. Thus, the initiating steps of the malignant B-cell transformation concern erroneous V(D)J rearrangement. Recurrent translocations involving the IgH or IgL locus and observed in B-cell lymphoproliferative disorders are shown in table 1, in relation to their biologic repercussions in disease entities concerned.

Disease Entity	IgH Translocation	Gene Involved	Biologic Consequences
MCL/MM	t(11;14)	Cyclin D1 encoded by CCND1	Regulator of CDKs CDK4/CDK6 required for cell cycle transition G1→S
FL	t(14;18)	Bcl2	Antiapoptotic
MM	t(4;14)	FGFR3	Signal transduction, pathways activation, cell proliferation regulation & differentiation
MM	t(6;14)	Cyclin D3	Cell cycle: G1→S transition
MM	t(14;20)	MAFB	Transcription factor, lineage specific hematopoiesis regulation
MM	t(14;16)	c-MAF	Cell cycle Stimulation. Promote interactions of tumor & stromal cells
BL/MM	t(8;14)	myc	Transcription factor, cell proliferation, differentiation, apoptosis, stem cell self renewal

Table 1. Main Recurrent Translocations Involving The IgH Locus

Monoclonal gammopathy of undetermined significance (MGUS) is a pro-neoplastic condition that may evolve into multiple myeloma (MM) or other B-cell lymphoproliferative disorders. MGUS represent a first step in the development of monoclonal diseases while the progression of MGUS to MM or other entities may be secondary to a random second genetic event. Several studies indicate that the majority of IgH locus aberrations reported in MM are already present in MGUS, favoring the hypothesis that these are early genetic events in the progression leading to MM [23].

In MM, the most frequent partners in reciprocal translocations involving the IgH locus on chromosome 14q32, are 11q13 (15%), 4p16 (5%), 16q23 (5%), 21q12 (2%) and 6p21 (2%); two additional partners are also found rarely 12p13 (<1%) and 8q24 (<1%). Thus, the aforementioned translocations may deregulate seven oncogenes involved, CCND1, CCND2, CCND3, MAF, MAFB, MAFA and FGFR3/MMSET [24]. The overall rate of 14q32 translocations increases with disease progression and reaches 90% in advanced tumors. Light chain translocations are rather rare in MM, particularly Igκ, which seem to be very infrequent [25]. Changes in the expression of gene subsets could be partly responsible for disease heterogeneity, as well as for further disease transformation. Moreover, with the 11q13 partner, constitutive upregulation of cyclin D1 results, deregulating the cell cycle [26]; t(11;14) is accompanied with a higher frequency of CD20 expression, hyposecretory disease and λ light chain usage. This subtype is increasingly encountered in AL amyloidosis, with or without MM, and in the rare IgM MM

and is associated with favorable outcome. Translocation $t(4;14)(p16;q32)$, is cryptic because of its telomeric location [27] and has been associated with IgA isotype, λ chain usage, deletion or monosomy of chromosome 13, immature plasma morphology, more aggressive disease and shortened survival. It leads to deregulation of fibroblast growth factor receptor 3 (FGFR3) gene on der(14) and of Multiple Myeloma SET (MMSET) domain gene on der(4); the latter may be a critical transforming event. $t(4;14)$ was found characterized by deregulation of chromatin organization, actin filament and microfilament movement [28].

The $t(14;16)(q32;q23)$ leads to the dysregulation of the c-maf oncogene; it is more frequently encountered in IgA isotype and is associated with chromosome 13 deletion whereas $t(14;20)(q32;q11)$ results in maf-B deregulation that like c-maf is a basic zipper transcription factor. The clinical significance of these rare IgH translocations is unknown and under investigation. However, the oncogenic process is continually going on during disease course and secondary IgH translocations can be observed such as those involving the myc oncogene (8q24), that are associated with advanced and aggressive disease. Especially in patients with cytogenetically high-risk disease, more changes are observed, including heterogeneous clonal mixtures with shifting predominant competitive clones [29].

It is interesting to observe that the abnormalities observed are not disease specific and can occur in different B-cell disorders in which they may confer different phenotypes, suggesting a role for additional factors [24].

A hallmark of Burkitt lymphoma (BL) is the expression of the myc oncogene, which has an essential role in cell proliferation, cell growth, protein synthesis, metabolism and apoptosis [30]. myc deregulated expression arises from $t(8;14)(q24;q32)$, juxtaposing myc to the IgH locus, in 80% of cases, whereas in the remaining, myc is translocated to the κ - (2p12), or λ - (22q11) light chain respectively. In endemic BL, most myc/IgH breakpoints originate from aberrant somatic hypermutation, in contrast to sporadic cases where the translocation mostly involves the Ig switch regions of the IgH locus at 14q32. The discrepancies are perhaps due to differences in Epstein Bar Virus positivity between endemic and sporadic forms [31]. myc translocations are not completely specific for BL and have been reported in other B-cell entities.

Almost 70% of mantle cell lymphoma (MCL) patients are genetically characterized by the chromosomal translocation $t(11;14)$. In several cases, patients also have point mutations and / or deletion of the ATM (ataxia telangiectasia mutated) gene. In addition, blastic forms or subtypes with more aggressive clinical behavior, may have additional mutations in genes that act as negative regulators of the cell cycle such as p16, p18 and p53 [32]. Rarer MCL cases are negative for cyclin D1, lack $t(11;14)$ and stand out of the usual clinical picture of MCL [33]; in such cases, cyclin D2 or cyclin D3 are overexpressed, a different permutation $t(2;12)(p12;p13)$ which connects cyclin D2 to the IgL-k locus may be present; it does not cause loss or quantitative disorder of genetic material, but at a molecular level, reconnecting two chromosomal regions can disrupt important genetic sequences, causing inactivation or gene mutation. Moreover, in this permutation, the protooncogene PRAD1 (Parathyroid Adenomatosis 1, or bcl1) which is normally found on chromosome 11, is swapped in the heavy chain Ig gene on chromosome 14 [34]. The resulting oncogene bcl1/IGH encodes cyclin D1 that is an important cell cycle regulator, particularly during the transition from the G1 to the S phase (the same

applies for cyclins D2 and D3). Under normal conditions, cyclin D1 acts through its interaction with cyclin dependent kinases (CDKs). CDKs are enzymes that add phosphate groups to protein-targets in order to make them inactive. The resulting complexes CDK4-D1 and CDK6-D3, promote the progress to cell cycle phase S, resulting in an uncontrolled proliferation.

Follicular lymphoma (FL) is characterized by the presence of chromosomal translocation t(14;18), which promotes protein bcl2 overexpression that in turn, leads to the suspension of apoptosis and survival increment of B cells that harbor the translocation. Less commonly, bcl2 is deregulated by translocation to the Igk t(2;8) and Igλ t(8;22) loci [35]. The t(14;18) is apparently mediated by the RAG recombinase proteins, which cleave at J segments in the IgH locus and at an unusual non B form DNA structure in bcl2. These B cells undergo an epigenetic reprogramming which, in conjunction with the acquisition of additional events, leads to FL development. The t(14;18)(q32;q21) may also be observed in diffuse large B cell lymphoma (DLBCL) [36] and in non-gastric MALT lymphomas. It brings the MALT1 gene under the control of the IGH enhancer [37].

3. Monoclonal immunoglobulins characteristics

3.1. Ig synthesis, secretion and metabolism

The IgH locus contains a region of 40-50 functional variable (VH), 27 diversity (DH) and 6 joining (JH) gene segments which is flanked by exons encoding the Ig constant regions (Cμ, Cδ, Cγ3, Cγ1, Cα1, Cγ2, Cγ4, Cε and Cα2). The Igκ locus contains 34-38 functional Vκ and 5 Jκ gene segments and one exon encoding the constant region of Igκ (Cκ). The Igλ locus comprises 29-30 functional Vλ and 4 functional Jλ-Cλ combinations [16]. Consequently, one of about fifty functional V_H, another of thirty D, and one of six J_H genes and, in the same way, one of thirty V_L and one of four J_L genes will be used. It appears that there are nearly 200 functional heavy and light chain gene segments that give rise to combinations of gene products, allowing the production of more than 5×10^7 antibodies with different unique variable end antigen combining sites [15;38;39]. Independently of the initiating stimulus, partly due to the aberrant Ig locus translocations and the putative activation or silencing of genes in monoclonal diseases, the cell starts to synthesize Ig following the variable domain rearrangement. On the coding DNA strand, the gene segments for the formation of the variable and the constant domains of the heavy chain are in order 5' VDJ-μ-δ-γ₃-γ₁-α₁-γ₂-γ₄-ε-α₂-3'. The RNA polymerase binds to the template strand of DNA and starts reading in 3' to 5' direction adding nucleotides to the 3' end of pre-mRNA transcript. Alternative splicing of the pre-mRNA brings together the VDJ variable domain and constant domain segments leading to the formation of the mRNA heavy Ig chain. As this procedure occurs in order, initially VDJs will get together with μ constant domain leading to the synthesis of heavy IgM component. This will bind with a light chain forming an IgM molecule. Thus, in order, cells make at first IgM, then IgD, IgG₃, IgG₁, IgA₁, IgG₂, IgG₄, IgE and IgA₂ that consist of the same variable domains but different constant domains due to alternative splicing and giving them different specific properties [40].

Light chains are synthesized in parallel to the heavy chain partner. However, an excess of light chains is produced, that if remained unbound to a heavy Ig component, will enter the blood and the extravascular compartment and circulate as free light chains (FLC). In patients with plasma cell dyscrasias (PCD) and B-cell lymphoproliferative disorders, homogeneous serum total Ig molecules (intact Ig) and serum FLCs (sFLCs) are secreted by the malignant clone [40].

sFLCs are rapidly cleared (2-6hrs) and metabolized by the kidney although trace quantities (1-10mg/L) can be found in the urine, produced by the lower urinary tract mucosa. With regard to intact Ig, IgA and IgM are cleared by pinocytosis and have constant half lives of 5-6 days while IgG has a concentration dependent variable half life, ranging from days to weeks or even months. Briefly, IgG is ingested by reticulo-endothelial cells by pinocytosis, but inside the endosome, it is bound by a recycling receptor called neonatal (FcRn) receptor and recycled back to the surface to be released. This process can occur many times and extends the half lives of both IgG and albumin, as FcRn binds to both IgG, via the constant domain, and albumin non-competitively [40]. When there is a large amount of IgG (as can be found in diseases such as IgG MM) the receptor becomes saturated and the half life of IgG is shorter.

3.2. Ig structure

Antibodies are the secreted form of the BCR, the simple symmetrical structure is conserved through the 5 immunoglobulin classes which are defined by their heavy chain amino acid sequences (γ , α , μ , δ and ϵ) although in MM IgM, IgD and IgE monoclonal proteins are rare. There is further subclass division for γ ($\gamma 1$, $\gamma 2$, $\gamma 3$, $\gamma 4$) and α ($\alpha 1$ and $\alpha 2$) immunoglobulin classes. Amino acid sequence analysis of the 5 immunoglobulin classes showed that each was based upon the same repeating structure, 2 identical light chains (~25kDa in size, 211-217 amino acids) and 2 identical heavy chains (~50kDa in size, 450-550 amino acids depending upon the class of heavy chain). Each of the immunoglobulin constituent proteins are constructed of β pleated sheets, which form the β barrel (Figure 2, A κ FLC molecule showing the constant region (left), and the variable region (right) with its alpha helix (red). (Courtesy of J Hobbs). Whilst there are obvious similarities between the different classes of immunoglobulin these structures are still being resolved and understood. IgG can be divided into 3 subunits, two identical fragment antigen binding arms (Fab) and an crystallizable (Fc) stem. Furthermore, within each subclass the hinge region shows differences both in the number of amino acids and the flexibility of the protein. More elegant electron tomography imaging of this molecule clearly shows its globular nature which perhaps gives a better indication of the protein structure. Serum IgA is predominantly a monomer, but dimeric forms can be found with J chain linkers. Solution scattering modelling of the two subclasses suggests a structure similar to IgG for IgA2 immunoglobulins, however IgA1 proteins appear to have a flattened "T" shaped structure. The traditional 2 dimensional representation of immunoglobulins belies their globular and highly variable nature, which may wrongly support the assumption that such molecules are simply quantified.

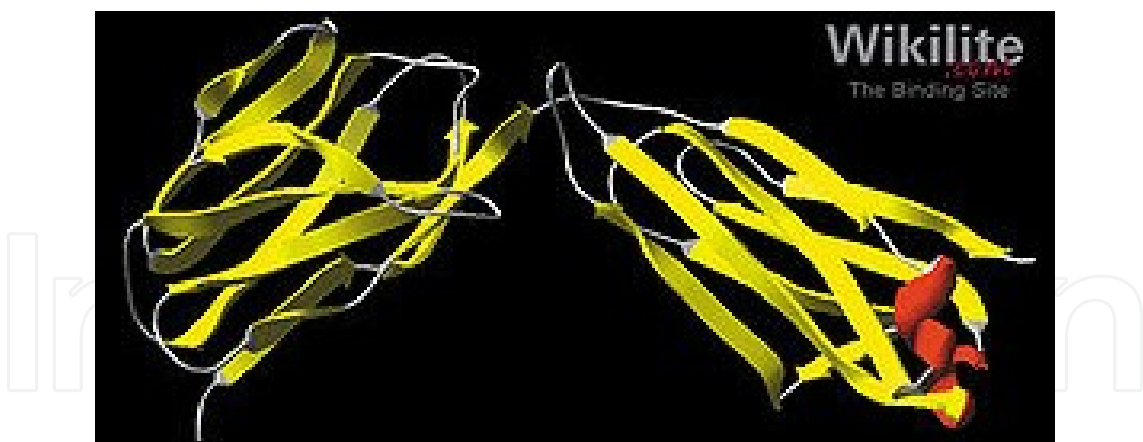


Figure 2. β pleated sheets of the kappa free light chain

3.3. Ig function

In normal conditions, the Ig or Ab (antibody) recognizes a unique part of the foreign target or antigen, called an epitope [40;41]. Each tip of the "Y" of an antibody contains a paratope (a structure analogous to a lock) that is specific for one particular epitope (similarly analogous to a key) on an antigen, allowing these two structures to bind together with precision. Using this binding mechanism, an antibody can tag a microbe or an infected cell for attack by other parts of the immune system, or can neutralize its target directly. Antibodies contribute to immunity in three ways: they prevent pathogens from entering or damaging cells by binding to them; they stimulate removal of pathogens by macrophages and other cells by coating the pathogens; and they trigger their destruction by stimulating other immune responses such as the complement pathway [42-44].

The five major Ab classes present complementary functions are shown in Table 2.

Immunoglobulin	Major Function
IgM	Main Ig during Primary Response (Early antibody). Fixes Complement (most effectively).
IgG	Main Ig during Secondary Response (late antibody). Opsonization. Fixes Complement. Neutralizes Toxins, Viruses.
IgA	Secretory mucosal Ig Prevents invasion from gut mucosa.
IgE	Immediate Hypersensitivity. Mast cell and Basophil reactions. Activates Eosinophils in helminth infection.
IgD	Function Unknown. Mostly on the Surface of B cells (B cell receptor).

Table 2. Major Functions of Antibodies Classes

Monoclonal Igs are not secreted after antigen exposure and do not contribute to combat pathogens; in fact humoral immunity is impaired because monoclonal plasma cells proliferate in detriment of normal Igs. Thus, in B-cell lymphoproliferative disorders, profound polyclonal hypogammaglobulinaemia can be observed leading to the inability to fight infections.

In some cases, the monoclonal Ig can have other effects, such as the ability to agglutinate red cells (cold agglutinin disease), to act as auto-antibody (autoimmune haemolytic anaemia), to aggregate at low temperatures (cryoglobulinemia), cause increased viscosity (Waldenström's macroglobulinemia), to deposit in tissues with resulting organ dysfunction (AL amyloidosis or immunoglobulin deposition diseases), and to cause peripheral neuropathy (MGUS, WM, AL amyloidosis, POEMS syndrome) [45].

3.4. Monoclonal immunoglobulin detection and quantification

Monoclonal intact immunoglobulin is routinely detected by serum protein electrophoresis (SPEP), the heavy chain class identified by immunofixation (IF) and quantified by SPEP-densitometry or nephelometry. Guidelines recommend SPEP to monitor monoclonal immunoglobulin concentrations as markers of response and relapse. However, SPEP quantification can be inaccurate at low concentrations (10g/L), can be difficult when the M-Ig co-migrates with other serum proteins (commonly IgA and IgM isotypes), when monoclonal immunoglobulins are produced by multiple small clones and is not suitable for sFLC quantification. Furthermore, poor linearity of SPEP at high concentrations and the variable catabolism of monoclonal IgG can make assessment of the serum load inaccurate. To aid patient monitoring international guidelines (IMWG 2011 consensus) recommend the use of total Ig nephelometric assays. At gross concentrations these assay are suitable tools to monitor patients; however, as they are unable to distinguish between the monoclonal and polyclonal Igs they will be insensitive as the Ig concentration approaches the normal range. One potentially useful addition to the laboratorian's armamentarium to overcome these issues are the newly developed heavy / light chain (HLC) immunoassays targeting the unique junctional epitope between the light chain (CL) and heavy chain (CH1) constant region of immunoglobulin, enabling the separate quantification of the different immunoglobulin classes i.e. HLC-IgG κ , -IgG λ , -IgA κ , -IgA λ , -IgM κ and -IgM λ . Measuring the molecules in pairs with this method enables the calculation of a ratio of the involved/uninvolved-polyclonal Igs (HLCR) [46-48] in the same manner as sFLC κ/λ ratios (FLCR).

SPEP quantification of sFLC is inaccurate and for more than 150 years monoclonal FLC measurements relied upon urinalysis. Collection, handling, renal function and variable light chain biochemistries make this a less than ideal medium for analysis. In the last 10 years the introduction of sheep based, polyclonal immunoassays for the quantification of sFLC κ and λ have changed the paradigm for FLC measurement. Briefly, polyclonal sheep antisera target κ and λ epitopes that are not available when the light chains are bound to their heavy chain partners [49]. As previously discussed FLCs are not homogeneous proteins and have significant genetic differences, particularly in the case of λ FLC, making the use of polyclonal antibodies (rather than monoclonal) necessary to ensure recognition of all FLC clones. The paired tests enable quantification of sFLC within and below the normal range which leads to

the identification of subtle monoclonal clones [87], below the sensitivity of SPEP and the qualitative IFE methods.

Intact Ig molecules, due to their size, are not filtered and excreted in the urine. Their presence in urine indicates glomerular damage and is usually part of the nephrotic syndrome that can accompany some monoclonal diseases (amyloidosis). If a simple urinalysis to identify protein in the urine gives a positive result, a 24-hour urine collection is required for urine IF. On the contrary, sFLCs that are much smaller, are freely filtered, excreted in the urine and metabolized in the urinary tract. During the initial stages of a plasma cell disorder, they are produced in small amounts that are entirely filtered by the urinary system; the majority is metabolized while small amounts may be excreted in the urine. Consequently, a negative serum IF may result while urine protein electrophoresis and IF may be positive. Urine test is not required for follow up due to the “paralogue phenomenon” of the sFLC. As the disease progresses the sFLC cause renal damage and decreased excretion from the kidneys leading eventually to decreased levels in the urine. If only urines are tested for follow up, low levels of sFLC could be found leading, in case of relapse, to wrongly consider disease improvement. In addition, during treatment, the reversal of renal damage will cause more excretion of sFLC to the urine, finding that should not be interpreted as disease progression.

3.5. Implications of monoclonal Ig in diseases

Monoclonal Ig, as measured by total Ig quantification, or more recently FLC or HLC, may contribute to diagnosis, response evaluation, disease monitoring or prognostication in plasma cell dyscrasias and B-cell lymphoproliferative disorders (Table 3).

3.5.1. Monoclonal gammopathy of undetermined significance

Monoclonal gammopathy of undetermined significance (MGUS) is an asymptomatic plasma cell dyscrasia that is present in more than 3% of the general white population older than age 50. It has an average multiple myeloma progression risk of about 1% per year [51]. The entity was first described by Waldenström in 1960 after abnormal narrow hypergammaglobulinemia bands were noted in the serum of healthy individuals on SPEP [52]. In 1978, Kyle introduced the term “monoclonal gammopathy of undetermined significance” after observing that asymptomatic patients with monoclonal protein have a higher risk of developing multiple myeloma, Waldenström macroglobulinemia, light-chain amyloidosis or related disorders [53]. Since then definition of MGUS has undergone several adaptations but always, paraprotein presence represented the backbone of its characterization.

In the updated 2010 IMWG diagnostic criteria, the definition of MGUS includes the presence of a serum monoclonal protein <3 g/dL, <10% clonal BM plasma cells infiltration and absence of end-organ damage (CRAB criteria of multiple myeloma) [54].

Over the last years, 3 distinct clinical subtypes of MGUS have been recognized: non-IgM MGUS, IgM MGUS and light-chain MGUS [55;56]. The best characterized MGUS subtype is non-IgM MGUS. Paraprotein isotypes of non-IgM MGUS patients can be further categorized into IgG (69%), IgA (11%) and biclonal (3%) [57]. Furthermore, IgD and IgE consist just a small

	Diagnostic Purposes	Prognosis	Staging	Response Evaluation	Monitoring
Total Ig					
MGUS	√	√		-	√
MM					
1. smoldering	√	-	√ ^{D-S}	-	√
2. LC	-	-	-	-	-
3. non-secretory	-	-	-	-	-
4. intact Ig MM	√	-	√ ^{D-S}	√	√
Solitary Bone Plasmacytoma	-	-	-	√*	-
AL Amyloidosis	-	-	-	√*	-
WM	√	-	√**	√	√
CLL	-	-	-	-	-
B-NHL	-	-	-	-	-
sFLC/sFLCR					
MGUS	√	√	√	-	√
MM					
5. smoldering	√	√	-	-	√
6. LC	√	√	-	√	√
7. non-secretory	√***	√***	-	√***	√***
8. intact Ig MM	-	√	-	√	√
Solitary Bone Plasmacytoma	-	√***	-	-	-
AL Amyloidosis	√	√	√	√	√
WM	-	√	-	?	?
CLL	-	√	√ [±]	-	-
B-NHL	?	?	-	-	-
HLC/HLCR					
MGUS	-	√	-	-	?
Intact Ig MM	-	√	-	√	√
WM	-	√	-	?	√
AL Amyloidosis	-	?	-	-	-

√:useful, -: not useful, ?: unknown, √^{D-S}: for Durie and Salmon Staging, *If present at diagnosis,** IgM level is included in the IPSS-WM, ***when abnormal sFLC/sFLCR is observed, its evaluation is useful; ±included in recently proposed CLL staging [50].

Table 3. Contribution of Total Ig, sFLC/sFLCR and HLC/HLCR Measurements In Plasma Cell Dyscrasias and B-cell Lymphoproliferative Disorders.

portion of all non-IgM MGUS cases [51]. Malignant transformation of non-IgM MGUS approximates 1% per year and typically develops into multiple myeloma rather than lymphoproliferative disorders [57]. IgM MGUS accounts for about 17% of all MGUS cases. It tends to progress to Waldenstrom macroglobulinemia or other lymphomas [58]. Finally, light-chain MGUS is characterized by the absence of intact IgM protein and the presence of monoclonal FLC characterized by a skewed FLC ratio, due to the increased levels of the monoclonal FLC

[59]. This last type is not frequently identified because asymptomatic patients are rarely tested with FLC assays; light chain MGUS' frequency is estimated at about 20% of cases.

Based on available clinical markers, two major predictive risk models of MGUS progression have been established by the Mayo clinic and the Spanish study group [55]. The Mayo clinic model indentifies 3 major risk factors: abnormal sFLC ratio, presence of non-IgG monoclonal Ig and monoclonal protein ≥ 15 g/l [59]. At 20 years of follow-up, the absolute risk of progression for MGUS patients with 0, 1, 2, and 3 risk factors is 5%, 21%, 37% and 58% respectively [59]. The Spanish study group proposes multiparametric flow cytometry as a tool to indentify aberrant plasma cell populations [60]. In addition, a recent study [61] showed that suppression of uninvolved immunoglobulin in MGUS, as detected by suppression of the isotype-specific heavy and light chain (HLC-pair suppression), is an independent risk factor for progression to malignancy. Uninvolved Ig suppression, occurring several years before malignant transformation takes place, offers a new perspective in early detection or even prediction of MGUS progression.

Monoclonal Ig is also the central marker used for MGUS patients follow-up. Moreover, patients should be followed performing SPEP, Ig and FLC quantification, at a frequency that depends on their risk-group.

Finally, special attention should be given to associations between MGUS and numerous diseases that are commonly encountered in clinical practice, because these may be related to underlying mechanisms with relevance in disease pathogenesis. In a retrospective cohort study of more than 4 million individuals, elevated risks of MGUS and MM were associated with broad categories of autoimmune, infectious, and inflammatory disorders but not allergies [62]. Systemic lupus erythematosus (SLE), a multisystem autoimmune disease characterized by profound B cell hyperactivity, autoantibody formation, and hypergammaglobulinemia, has been associated with MGUS, although the latter is not clearly a manifestation of disease activity and its significance remains to be elucidated [63]. Two possible mechanisms have been proposed for the aforementioned correlation of the two nosological entities. The first hypothesis claims that B cell hyperactivity in SLE favours the escape of B cell clones from the normal regulatory mechanisms. An alternative hypothesis is that defective immunological surveillance, predisposing to malignancies in general, promotes the development of MM and/or its precursor state MGUS. Concerning rheumatoid arthritis (RA), several studies have indicated a direct correlation of the disease with MGUS presence. More specifically, 1.7% of patients with classical RA and high-titre rheumatoid factor present with MGUS [64].

3.5.2. Multiple myeloma

Multiple myeloma (MM) is an heterogeneous PCD with a wide range of clinical manifestations and outcomes, affecting terminally differentiated B-cells and characterized by bone marrow infiltration by monoclonal plasma cells secreting a monoclonal Ig. The disease might be asymptomatic, requiring only follow-up, or symptomatic and accompanied by fatigue, bone pains or spontaneous fractures, renal failure, recurrent infections or other morbid symptoms. In such cases treatment is immediately needed to prevent if possible irreversible organ damage. Paraprotein presence and amount are included into the diagnostic criteria [65-67]. The

diagnostic criteria for smoldering (asymptomatic) multiple myeloma is a serum M protein level of $\geq 3\text{g/dL}$, $\geq 10\%$ BM plasma cells infiltration, and no related organ or tissue impairment (including bone lesions) or symptoms and the diagnostic criteria for symptomatic multiple myeloma is M protein (serum or urine) presence, BM plasma cell infiltration of $\geq 10\%$ or histologically proven plasmacytoma, and myeloma-related organ or tissue impairment [68], further characterized by the CRAB criteria of multiple myeloma for end-organ damage, consisting of hypercalcemia (calcium level $> 11.5\text{mg/dL}$), renal failure (serum creatinine $> 2.0\text{mg/dL}$ or estimated creatinine clearance $< 40\text{ mL/min}$), anemia (hemoglobin level $< 10\text{ g/dL}$ or hemoglobin level at least 2g/dL below the lower normal limit) and bone lesions (lytic lesions, severe osteopenia or pathologic fractures) [54]. sFLC measurements also are useful for diagnostic purposes, especially in light chain myeloma (LCM) and oligosecretory disease [69].

The evaluation of response to treatment is largely based on Ig decrease with complete response (CR) identified as negative IFE on both serum and urine, maintained for a minimum of 6 weeks [70]. In an attempt to improve response criteria, sFLCR was incorporated to the MM uniform response criteria [71] and its normalization along with immunohistological or immunophenotype confirmation of clonal disease absence, defined a deeper response, the stringent complete response (sCR). A better evaluation of the depth of response is important as the quality of response is correlated with treatment free and overall survival after treatment [72]. In the same way, relapse is established by an Ig increase on SPEP, total Ig quantification, sFLCs and more recently HLCs; all the aforementioned methods can therefore be used for disease monitoring [73]. An additional contribution of sFLC measurements for disease monitoring during follow-up of patients is that light chain only relapses may be observed, with the improvement of treatment modalities resulting in prolonged survival. Disease transformation characterized by light chain escape may occur, characterized by a shift in secretion from intact Ig to LC only in a subset of patients [74;75] that could be otherwise considered in plateau.

With regard to prognosis, although serum monoclonal Ig quantification was one of Durie and Salmon staging system's risk factors [76] and was included in older prognostic algorithms [77], it was subsequently not shown to be linked with MM aggressiveness and was not retained as a prognostic risk parameter [78]. However, paraprotein type was shown to influence survival; IgG patients being most favourable, followed by IgA while light chain MM patients had the worst prognosis [79]. The introduction of the new Ig-based biomarkers (sFLC/HLC) rehabilitate monoclonal Igs prognostic potential in MM. Thus, sFLC and sFLCR were shown predictive of outcome in all MM subcategories. Patients with smoldering myeloma and abnormal sFLCR were shown to have an increased progression risk while an adverse outcome was observed in patients with overt MM and increased sFLCR [80;81]. In addition, the combination of sFLCR and other markers of disease activity (LDH, $\beta 2$ -microglobulin, genetic abnormalities) or the International Score System (ISS) for MM, were reported to produce powerful prognostic models [78;82], although this is yet to be proven in the era of novel therapies [83]. Furthermore three groups showed simultaneously that HLC-IgG and -IgA ratios (HLCR) were predictive of a shorter overall survival [73;84] and progression-free survival [85].

3.5.3. AL amyloidosis

Systemic AL amyloidosis is characterised by the deposition of misfolded monoclonal light chains or their fragments in tissues or organs, leading to visceral dysfunction [86]. Symptomatology depends on the organ(s) involved and includes nephrotic syndrome, skin lesions, cardiomyopathy, demyelinating peripheral neuropathy, hepatomegaly, malabsorption syndrome, etc. Diagnosis is frequently difficult, in the (usual) absence of characteristic signs such as macroglossia or periorbital purpura. Physicians should be aware of the possible diagnosis of AL amyloidosis in patients with unexplained fatigue, and FLCR can aid in the differential diagnosis. In such a context sFLC measurements are useful and will be found increased in up to 94-98% of patients, even in the absence of any Ig monoclonal peak on serum electrophoresis or immunoelectrophoresis. However, diagnosis should be proven by involved tissue biopsy. Kidney is the most frequently involved organ while cardiac deposits are the most deleterious and related to shorter survival. AL amyloidosis may complicate MM or other PCD in less than 10% of cases.

sFLC levels concentrations at diagnosis are by themselves an adverse marker of survival in AL amyloidosis [88]. The addition of cardiac biomarkers to sFLC levels at diagnosis was shown highly predictive of patients survival [89] and a new prognostic staging system was built [90]; a score of 1 for each of three prognostic variables, namely cardiac troponin T (cTnT) (>0.025 ng/mL, N-terminal pro-B-type natriuretic peptide (NT-ProBNP) ($>1,800$ pg/mL), and FLC difference (FLC-diff) (>18 mg/dL), was used to divide patients into four stages (I, II, III, and IV) with scores of 0, 1, 2, and 3, respectively. The 5-year survival estimates produced for patients in stage I, II, III, and IV were 59%, 42%, 20%, and 14% respectively ($p<0.001$).

Preliminary data on HLC measurements in AL amyloidosis appear promising. In a subset of AL amyloidosis patients with no detectable serum or urinary monoclonal bands and a normal sFLC ratio, the HLC ratio was abnormal in 19% of cases, identifying 2 IgA κ , 3 IgA λ , and 4 IgG κ clones [91].

3.5.4. Waldenstroms Macroglobulinemia

Waldenstroms Macroglobulinemia (WM) is a lymphoplasmacytic lymphoma (LPL) [22] characterized by lymphoplasmacytic infiltration of BM and eventually other organs, and by the presence of a serum IgM monoclonal component. IgM paraprotein is mandatory to establish the diagnosis. In case of a biology proven LPL without IgM, the disease will be called just LPL, not WM.

WM is a rare disease entity that presents a wide range of clinical signs and symptoms including those due to the lymphoma (lymph nodes' swelling, organomegaly, bone marrow failure) and those due to the presence of the IgM paraprotein. IgM-related symptoms are hyperviscosity, autoimmune phenomena (peripheral neuropathy, haemolytic anaemia, thrombocytopenic purpura), cryoglobulinaemia, amyloidosis. Asymptomatic patients do not require treatment and usually enjoy a prolonged survival, while patients with aggressive symptomatic disease should be immediately treated with chemotherapy [92;93]. Evaluation of response is based on changes in serum IgM concentrations and other factors. Complete response (CR) is character-

ized by the disappearance of symptoms, of monoclonal serum IgM (by IF), and of monoclonal lymphoplasmacytes from all infiltrated sites, partial response by a serum IgM decrease by 50% or more while progressive disease (PD) by IgM increase; likewise, relapse after response is characterized by IgM increase [94]. With regard to staging and prognosis, serum IgM levels were included into currently used international prognostic staging system for WM (IPSS-WM) that co-evaluated 5 parameters: age above 65 years, haemoglobin below 11,5 g/dL, platelet counts below or equal to $100 \times 10^9/L$, β_2 -microglobulin above 3mg/L and IgM above 7 g/dL [95].

There are so far only preliminary results on the contribution of the new Ig-based biomarkers (sFLC and HLC) levels in WM patients at diagnosis. It was shown that sFLC may be increased and, in such cases, correlate with markers of disease activity, such as increased β_2 M, anemia [96] and low serum albumin levels. Patients with elevated sFLC presented shorter time to treatment [97] and adverse outcome [98]. Increased HLC-IgM were also found correlated with markers of disease activity such as bone marrow infiltration of more than 50% and low serum albumin levels while high HLCR correlated with shorter time to treatment [98;99].

3.5.5. *Chronic lymphocytic leukemia*

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in the Western world and presents a large range of clinical manifestations and a variable outcome. More than two thirds of the patients are asymptomatic at the time of diagnosis and may not require treatment for months or even years. For prognostic purposes, traditional Rai and Binet clinical staging systems are still in use but they do not apply perfectly in modern years. For patients needing treatment, underlying molecular alterations are important predictors of response; however, for the majority of CLL patients, life expectancy largely depends on time to first treatment [100], so reliable markers for time to treatment are needed.

It was shown that increased sFLC is the most common paraprotein observed in CLL, being found in almost half of the cases and that sFLCR abnormalities are present in a significant proportion of patients and identify those at risk of progressive disease [101;102].

More recently, increased polyclonal sFLC were also found to constitute an adverse marker for time to first treatment in CLL [103]. This finding was confirmed by Morabito et al that evaluated the sum of κ and λ sFLC levels and found that the prognostic impact of sFLC ($\kappa + \lambda$) value above 60.6 mg/mL was superior compared to FLCR and built a model based on four variables, namely sFLC ($\kappa + \lambda$) more than 60.6 mg/mL, Binet staging, ZAP-70, and cytogenetics and separated 4 patients' groups with different time to treatment [50].

3.5.6. *Other plasma cell dyscrasias & B-cell non Hodgkin's lymphomas*

In the other PCD, Ig contribution to diagnosis, prognosis and monitoring is restricted to bone solitary plasmacytoma and mainly concerns sFLC quantification that was shown predictive of evolution to MM [104]. With regards to B-cell non Hodgkin's lymphomas, abnormal Ig secretion, as observed mostly by the new Ig-based biomarkers (sFLC and HLC) levels, the clinical significance of which remains for the time being, under investigation [105], although increasing evidence of sFLCs prognostic role are emerging in these diseases [106;107].

4. Conclusions

Paraprotein presence is the hallmark of monoclonality. Knowledge of biologic mechanisms that lead to monoclonality has allowed understanding of malignant B-cell origin and B-cell neoplasms pathophysiology. New methods for the precise detection and quantification of monoclonal Ig have opened interesting clinical applications concerning patients diagnosis, monitoring and prognostication.

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